TITLE OF THE INVENTION

A NOVEL BACILLUS *THURINGIENSIS* STRAIN, CRYSTAL GENE AND CRYSTAL PROTEIN AND USES THEREOF

FIELD OF THE INVENTION

[0001] The present invention relates to a novel *Bacillus thuringiensis* strain, crystal gene and crystal protein and uses thereof. More specifically, the present invention is concerned with a novel *Bacillus thuringiensis*, novel Cry31 protoxin and toxin, nucleotide sequences encoding same and anti-cancer therapeutic applications for the toxin.

BACKGROUND OF THE INVENTION

[0002] *Bacillus thuringiensis* has been known for years for coding for δ-endotoxin crystal proteins. A large variety of endotoxins have been described and characterized, many of them having reported insecticidal activities. Most of these have molecular weights in the range of 130-140kDa and 65-80kDa (Schnepf *et al.*, 1998). Recently, a novel endotoxin protein has been identified and designated Cry31Aa1 (also called parasporin) (Mizuki *et al.*, 2000). It is an 81 KDa protein encoded by a 2169 bp gene that has been characterized as having a selective activity as a human Leukemic Cell-Recognizing Protein (Mizuki *et al.*, (1999) and (2000)). No other member of this novel family of endotoxin has yet been reported.

[0003] It is therefore an object of the present invention to provide a new *bacillus thuringiensis* strain expressing a new member of this novel family of δ-endoxins displaying advantageous cytotoxicity against human cancer cells.

SUMMARY OF THE INVENTION

[0004] More specifically, in accordance with the present invention, there is provided a novel *Bacillus thuringiensis* strain, named M15, a novel 83-kDa crystal protein δ-endotoxin assigned the designation Cry31Aa2 by the *Bacillus thuringiensis* Pesticide Crystal Protein Nomenclature Committee and displaying cytotoxicity against certain human cancer cells.

[0005] According to a first aspect of the present invention, there is also provided a biologically pure culture of a microorganism strain comprising all of the identifying characteristics of a *Bacillus thuringiensis* strain deposited at the International Depository Authority of Health Canada in Winnipeg under accession number IDAC010201-5, or a mutant thereof derived from said strain.

[0006] According to a second aspect of the present invention, there is also provided An isolated nucleic acid molecule comprising a polynucleotide sequence selected from the group consisting of: (a) a nucleotide sequence encoding a polypeptide comprising the complete amino acid sequence in SEQ ID NO: 2; (b) a nucleotide sequence encoding a polypeptide comprising the complete amino acid sequence in SEQ ID NO: 8; (c) a nucleotide sequence encoding a polypeptide comprising the complete amino acid sequence in SEQ ID NO: 12, with the proviso that said nucleotide sequence does not encode the amino acid sequence in SEQ ID NO: 18; (d) a nucleotide sequence encoding a polypeptide comprising the complete amino acid sequence in SEQ ID NO: 13, with the proviso that said nucleotide sequence does not encode the amino acid sequence at positions 232 to 723 of SEQ ID NO: 18; (e) a nucleotide sequence encoding a polypeptide comprising the complete amino acid sequence in SEQ ID NO: 14, with the proviso that said nucleotide sequence does not encode the amino acid sequence in SEQ ID NO:

18; (f) a nucleotide sequence encoding a polypeptide comprising the complete amino acid sequence in SEQ ID NO: 15, with the proviso that said nucleotide sequence does not encode the amino acid sequence at positions 232 to 723 of SEQ ID NO: 18; (g) a nucleotide sequence encoding a polypeptide comprising the complete amino acid sequence of a crystal protein contained in the *Bacillus thuringiensis* strain deposited at the International Depository Authority of Health Canada in Winnipeg under accession number IDAC010201-5; (h) a nucleotide sequence encoding a crystal protein comprising the complete amino acid sequence in SEQ ID NO: 10; (i) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 1; (j) a nucleotide sequence comprising the sequence set forth in SEQ ID NO: 9; (k) a nucleotide sequence encoding a crystal protein comprising the sequence set forth in SEQ ID NO: 11; (l) a nucleotide sequence encoding a crystal protein having at least 94% identity with the complete amino acid sequence in SEQ ID NO: 2, with the proviso that said nucleotide sequence does not encode the amino acid sequence in SEQ ID NO: 18; (m) a nucleotide sequence encoding a crystal protein having at least 97% identity with the complete amino acid sequence in SEQ ID NO: 8 with the proviso that said nucleotide sequence does not encode the amino acid sequence from position 232 to 723 of SEQ ID NO: 18; (n) a nucleotide sequence encoding a crystal protein cytotoxic against at least one human cancer cell, said nucleotide sequence having at least 98% identity with the complete sequence set forth in SEQ ID NO: 9, with the proviso that said nucleotide sequence does not encode the amino acid sequence from position 232 to 723 of SEQ ID NO: 18; (o) a nucleotide sequence completely complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g), (h), (i) (j), (k), (l), (m) and (n); and (p) a nucleotide sequence which hybridizes under high stringency conditions to any of the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g), (h), (i) (j), (k), (l), (m), (n) and (o).

[0007] An isolated polypeptide comprising a sequence selected from the group consisting of: (a) an amino acid as set forth in SEQ ID NO: 2; (b) an amino acid sequence in SEQ ID NO: 8; (c) an amino acid sequence of a crystal protein contained in the *bacillus thuringiensis* strain in the deposit at the International Depository Authority of Health Canada in Winnipeg under accession number IDAC010201-5; (d) a crystal protein comprising the amino acid sequence in SEQ ID NO: 10; (e) a crystal protein having at least 94% identity with the complete amino acid sequence in SEQ ID NO: 2, with the proviso that said crystal protein is not constituted of SEQ ID NO: 18; (f) a crystal protein having at least 97% identity with the complete amino acid sequence in SEQ ID NO: 8, with the proviso that said crystal protein is not constituted the amino acid sequence at positions 232 to 723 of SEQ ID NO: 18; (g) a crystal protein cytotoxic against at least one human cancer cell and encoded by a nucleotide sequence having at least 98% identity with the complete sequence in SEQ ID NO: 9, with the proviso that said nucleotide sequence does encode the amino acid sequence at positions 232 to 723 of SEQ ID NO: 18.

[0008] According to an other aspect of the present invention, there is also provided a recombinant vector comprising an isolated nucleotide sequence of the present invention, a recombinant host cell same, a method for making same comprising inserting such isolated nucleic acid molecule in a vector.

[0009] According to an other aspect of the present invention, there is also provided a recombinant method for producing a cytotoxic polypeptide, comprising culturing the host cell under conditions such that the polypeptide is expressed and recovering said polypeptide.

[0010] According to an other aspect of the present invention, there is also provided an isolated antibody that binds specifically to a polypeptide of the present invention.

[0011] According to an other aspect of the present invention, there is also provided a method of modulating the level of cry31Aa2 active protein in a cell comprising a modulation of the level or activity of the sequence SEQ ID NO: 8.

[0012] According to an other aspect of the present invention, there is also provided a method of using a polypeptide of the present invention for lysing a human cancer cell which according a specific embodiment of the present invention is selected from the group consisting of HELA, TCS, HL-60, Jurkat, and Hep-G2 cells.

[0013] According to an other aspect of the present invention, there is also provided a method of testing the cytotoxicity of a polypeptide of the present invention against a candidate cancer cell comprising determining the EC50 of the polypeptide on the candidate cell, wherein the polypeptide is characterized as possessing cytotoxicity against the candidate cell if the EC50 of the polypeptide against the candidate cell is measurably lower than that against a normal T cell.

[0014] According to an other aspect of the present invention, there is also provided a method for lysing a human cancer cell comprising applying a cytotoxic amount of a polypeptide of the present invention on a human cancer cell.

[0015] According to an other aspect of the present invention, there is

also provided a method for obtaining a cytotoxic polypeptide comprising cleaving a polypeptide of the present invention with a protease able to cleave between a residue R and a residue I. In a specific embodiment, the protease is trypsin.

[0016] In order to provide a clear and consistent understanding of terms used in the present description, a number of definitions are provided hereinbelow.

[0017] Unless defined otherwise, the scientific and technological terms and nomenclature used herein have the same meaning as commonly understood by a person of ordinary skill to which this invention pertains. Generally, the procedures for molecular biology methods and the like are common methods used in the art. Such standard techniques can be found in reference manuals such as for example Sambrook *et al.* (1989, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) and Ausubel *et al.* (1994, Current Protocols in Molecular Biology, Wiley, New York).

[0018] The terminology "human cancer cell" as used herein refers to cells associated with at least one type of cancer. Without limiting the generality of this definition, this terminology includes the following cells and corresponding tissues, namely acetabulum: HT-1080; amnion: WISH; B-cells: NAGL-1; blood: J-111, IM-9, jurkat; bone: HOS, MG-63, MEG-01; bone marrow: A549; MEG-01; FS-1; brain: SF126, U-251, MG, Becker, Marcus, T98G, SK-MG-1, ONS-76, KNS, B2-17, no. 10, no. 11, KALS-1, KINGS-1, KS-1, KNS-81-FD, NMC-G1, GB-1, AM-38, YH-13; colon: WiDr, LoVo, CCD 841, CCD-33, Caco-2; embryonic limb: Miz-1; epidermoid: A-431; whole fetus: HE-1; foreskin: FLOW7000, Hs68, liver: Chang Liver, Alexander cells, HC, Hep-G2; lung:

MRC-5, MRC-9, HFL1, WI-38, Flow 2000, KNS-62; lymph node: GAK; lymphoblastoid: Namalwa; maxilla: Raji; melanoma: G-361, A2058; neuroblastoma: KP-N; ovary: RMG, RKN, RTSG, RMUG; peripheral blast: MTA; peripheral blood: RPMI 8226, HL-60, CCRF-SB, EB-3, RPMI 788, NC-37, MOLT-4, KU812, CCRF-CEM, CMK, NOMO, NKM-1, MEG-A2, TMD5, KAI3; pleural effusion: U-937; prostate: DU145, CEACAM-1; sympatho-adrenal cell: IMR-32, NB-1; umbilical cord: HUV-EC-C; uterine cervix: Ca Ski, HeLa, SKG, BOKU; uterine endometrium : SNG; uterus: SKN, NJG, SAWANO, TCS, UtSMC. The terminology "cancer cell" also refers herein to cells associated with non-human forms of cancer including Vero, COS-7 and NIH3T3 cells.

[0019] As used herein, a "biologically pure" strain is intended to mean the strain separated from materials with which it is normally associated in nature. Note that a strain associated with other strains, or with compounds or materials that it is not normally found with in nature, is still defined as "biologically pure." A monoculture of a particular strain is, of course, "biologically pure."

Nucleotides

[0020] Nucleotide sequences of the present invention are presented herein by single strand, in the 5' to 3' direction, from left to right, using the one letter nucleotide symbols as commonly used in the art and in accordance with the recommendations of the IUPAC-IUB Biochemical Nomenclature Commission.

[0021] The present description refers to a number of routinely used recombinant DNA (rDNA) technology terms. Nevertheless, definitions of selected examples of such rDNA terms are provided for clarity and consistency.

[0022] As used herein, "nucleic acid molecule", refers to a polymer of nucleotides. Non-limiting examples thereof include DNA (e.g. genomic DNA, cDNA), RNA molecules (e.g. mRNA) and chimeras thereof. The nucleic acid molecule can be obtained by cloning techniques or synthesized. DNA can be double-stranded or single-stranded (coding strand or non-coding strand [antisense]).

Protein expression

[0023] Prokaryotic expressions are useful for the preparation of large quantities of the Cry31Aa2 protoxine and toxine encoded by the *cry31Aa2* DNA sequence. These proteins can be purified according to standard protocols that take advantage of the intrinsic properties thereof, such as size and charge (e.g. SDS gel electrophoresis, gel filtration, centrifugation, ion exchange chromatography...). In addition, the protein of interest can be purified via affinity chromatography using polyclonal or monoclonal antibodies. The purified protein can be used for therapeutic applications in accordance with the methods and uses of the present invention.

Mutations, mutants and variants

[0024] As commonly known, a "mutation" is a detectable change in the genetic material which can be transmitted to a daughter cell. As well known, a mutation can be, for example, a detectable change in one or more deoxyribonucleotides. For example, nucleotides can be added, deleted, substituted for, inverted, or transposed to a new position. Spontaneous mutations and experimentally induced mutations exist. A mutant polypeptide can be encoded from this mutant nucleic acid molecule.

[0025] As used herein, a "mutant" of the novel strain of *Bacillus thuringiensis* of the present invention namely the M15 strain deposited under access no, IDAC010201-5 may or may not have the same identifying biological characteristics of the M15 strain, as long as the mutant produces a crystal protein that is cytotoxic against human cancer cells. Illustrative examples of suitable methods for preparing mutants and variants of the inventive microorganism strain include, but are not limited to: mutagenesis by irradiation with ultraviolet light or X-rays, or by treatment with a chemical mutagen such as nitrosoguanidine (N-methyl-N'-nitro-N-nitrosoguanidine), methylmethanesulfonate, nitrogen mustard and the like; gene integration techniques, such as those mediated by insertional elements or transposons or by homologous recombination of transforming linear or circular DNA molecules; and transduction mediated by bacteriophages such as P1. These methods are well known in the art and are described, for example, in J. H. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1972); J. H. Miller, A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1992); M. Singer and P. Berg, Genes & Genomes, University Science Books, Mill Valley, CA (1991); J. Sambrook, E. F. Fritsch and T. Maniatis, Molecular Cloning: A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); P. B. Kaufman *et al.,* Handbook of Molecular and Cellular Methods in Biology and Medicine, CRC Press, Boca Raton, FL (1995); Methods in Plant Molecular Biology and Biotechnology, B. R. Glick and J. E. Thompson, eds., CRC Press, Boca Raton, FL (1993); and P. F. Smith-Keary, Molecular Genetics of *Escherichia coli*, The Guilford Press, New York, N.Y. (1989).

[0026] Mutated strains derived from the M15 strain using known methods are then preferably selected or screened for improved cytotoxic crystal proteins production potential or for other desirable properties related to

their utility in expressing crystal proteins that are cytotoxic to human cancer cells. In a specific embodiment of the mutagenesis and screening approach to strain improvement, mutagenized cells are selected on the basis of their cytopathic effects or cytocidal activity on target cells and their spectrum of action.

[0027] The term "variant" refers herein to a protein or nucleic acid molecule which is substantially similar in structure and biological activity to the protein or nucleic acid of the present invention and includes a *cry31Aa2* nucleic sequence or the protein encoded by same having one or more mutations that does not affect its cytotoxic activity. In particular, a variant of the nucleotide or polypeptide sequence of the active portion of Cry31Aa2 possesses the ability to lyse human cancer cells including HeLa, TCS, HL-60, Jurkat and Hep-G2. The methods for determining whether a nucleotide or polypeptide sequence constitutes a variant of Cry31Aa2 include conducting an EC50 assay on a cancer cell against which the Cry31Aa2 active toxin displays cytotoxicity.

[0028] The functional derivatives of the present invention can be synthesized chemically or produced through recombinant DNA technology. All these methods are well known in the art.

Hybridization

[0029] "Nucleic acid hybridization" refers generally to the hybridization of two single-stranded nucleic acid molecules having complementary base sequences, which under appropriate conditions will form a thermodynamically favored double-stranded structure. Examples of hybridization conditions can be found in the two laboratory manuals referred above (Sambrook *et al.*, 1989, supra, and Ausubel *et al.*, 1989, supra) and are commonly known in the art. In the case of an hybridization to a nitrocellulose

filter, as for example in the well known Southern blotting procedure, a nitrocellulose filter can be incubated overnight at 65°C with a labeled probe in a solution containing 50% formamide, high salt (5 x SSC or 5 x SSPE), 5 x Denhardt's solution, 1% SDS, and 100 μg/ml denatured carrier DNA (e.g. salmon sperm DNA). The non-specifically binding probe can then be washed off the filter by several washes in 0.2 x SSC/0.1% SDS at a temperature which is selected in view of the desired stringency: room temperature (low stringency), 42°C (moderate stringency) or 65°C (high stringency). The selected temperature is based on the melting temperature (Tm) of the DNA hybrid. Of course, RNA-DNA hybrids can also be formed and detected. In such cases, the conditions of hybridization and washing can be adapted according to well known methods by the person of ordinary skill. Stringent conditions will be preferably used (Sambrook *et al.*,1989, supra). In most hybridizations, a 1% mismatching of bases will lower the melting temperature by 1-1.5°C (Sambrook *et al.*,1989, supra). Consequently, nucleotide sequences sharing 98% nucleotide identities with the *cry31Aa2* gene encoding the trypsin-activated portion of Cry31Aa2 will still hybridize with the *cry31Aa2* gene when the melting temperature is lowered by 3°C, respective to the most stringent conditions for hybridization between two identical *cry31Aa2* sequences. The term "high stringency" conditions refer herein to the conditions required for the hybridization of nucleotide sequences sharing at least 98% nucleotide identities with the *cry31Aa2* gene encoding the trypsin-activated portion of Cry31Aa2.

[0030] As used herein, "chemical derivatives" is meant to cover additional chemical moieties not normally part of the subject matter of the invention. Such moieties could affect the physico-chemical characteristic of the derivative (e.g. solubility, absorption, half life, decrease of toxicity and the like). Such moieties are exemplified in Remington's Pharmaceutical Sciences (1980). Methods of coupling these chemical-physical moieties to a polypeptide or

nucleic acid sequence are well known in the art.

Recombinant vectors

[0031] The term "recombinant DNA" as known in the art refers to a DNA molecule resulting from the joining of DNA segments. This is often referred to as genetic engineering. The same is true for "recombinant nucleic acid".

[0032] The term "vector" is commonly known in the art and defines a plasmid DNA, phage DNA, viral DNA and the like, which can serve as a DNA vehicle into which DNA of the present invention can be cloned. Numerous types of vectors exist and are well known in the art.

[0033] The term "expression" defines the process by which a gene is transcribed into mRNA (transcription), the mRNA is then being translated (translation) into one polypeptide (or protein) or more.

[0034] The terminology "expression vector" defines a vector or vehicle as described above but designed to enable the expression of an inserted sequence following transformation into a host. The cloned gene (inserted sequence) is usually placed under the control of control element sequences such as promoter sequences. The placing of a cloned gene under such control sequences is often referred to as being operably linked to control elements or sequences.

[0035] Operably linked sequences may also include two segments that are transcribed onto the same RNA transcript. Thus, two sequences, such

as a promoter and a "reporter sequence" are operably linked if transcription commencing in the promoter will produce an RNA transcript of the reporter sequence. In order to be "operably linked" it is not necessary that two sequences be immediately adjacent to one another.

[0036] Expression control sequences will vary depending on whether the vector is designed to express the operably linked gene in a prokaryotic or eukaryotic host or both (shuttle vectors) and can additionally contain transcriptional elements such as enhancer elements, termination sequences, tissue-specificity elements, and/or translational initiation and termination sites.

[0037] The DNA construct can be a vector comprising a promoter that is operably linked to an oligonucleotide sequence of the present invention, which is in turn, operably linked to a heterologous gene, such as the gene for the luciferase reporter molecule. "Promoter" refers to a DNA regulatory region capable of binding directly or indirectly to RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of the present invention, the promoter is bound at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter will be found a transcription initiation site (conveniently defined by mapping with S1 nuclease), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CCAT" boxes. Prokaryotic promoters contain -10 and -35 consensus sequences, which serve to initiate transcription and the transcript products contain Shine-Dalgarno sequences, which serve as ribosome binding sequences during translation initiation.

Recombinant host cell

[0038] A host cell or indicator cell has been "transfected" by exogenous or heterologous DNA (e.g. a DNA construct) when such DNA has been introduced inside the cell. The transfecting DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transfecting DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transfected cell is one in which the transfecting DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transfecting DNA. Transfection methods are well known in the art (Sambrook *et al.*, 1989, supra; Ausubel *et al.*, 1994 supra).

Method for identifying other cancer cells against which Cry31Aa2 is cytotoxic

[0039] In addition to the EC50 assay described herein, other assays may be used to determine the effects of Cry31Aa2 or other proteins encompassed by the present invention on human cancer cells. In particular, these effects may be observed by photonic microscopy. Furthermore, assays for detecting cytopathic effects can also be used for this purpose.

[0040] Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments thereof, given by way of example only with reference to the accompanying drawings.

[0041] The present invention seeks to meet these needs and other needs.

[0042] The present description refers to a number of documents, the content of which is herein incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

In the appended drawings:

[0043] Figure 1 illustrates in panel A) a phase-contrast micrograph of a lysed culture of *Bacillus thuringiensis* strain M15; in panel B, a transmission electron micrograph of *Bacillus thuringiensis* strain M15 containing a spore and a tightly bound parasporal inclusion;

[0044] Figure 2 shows a SDS-PAGE analysis of the parasporal inclusion protein(s) of *B. thuringiensis* strain M15;

[0045] Figure 3 is the nucleotide sequence of the translated portion of the *cry31Aa2* gene (SEQ ID NO: 1);

[0046] Figure 4 is the deduced amino acid sequence of the *cry31Aa2* gene (SEQ ID NO: 2);

[0047] Figure 5 shows a comparison of the deduced amino acid sequences of Cry31Aa2 (SEQ ID NO: 2) and Cry31Aa1 (SEQ ID NO: 18). The capital letters and dotted lines under the amino acid sequence of Cry31Aa2 (SEQ ID NO: 2) correspond to the difference and alignment gaps between the cry31Aa2 (SEQ ID NO: 2) and Cry31Aa1 (SEQ ID NO: 18). The asterisks under the Cry31Aa2 sequence indicate the identities between Cry31Aa2 (SEQ ID NO: 2) and Cry31Aa1(SEQ ID NO: 18);

[0048] Figure 6 shows a restriction map of the recombinant plasmid pYCP31A2 containing the *cry31Aa2* gene;

[0049] Figure 7 shows a transmission electron micrograph of a *B. thuringiensis* Cry^- B transformant expressing the *cry31Aa2* gene. S: spore; P: parasporal inclusion; Magnification : 20,000 X;

[0050] Figure 8 shows a SDS-PAGE analysis of the parasporal inclusion protein from a *B. thuringiensis* transformant expressing the crystal protein gene *cry31Aa2*;

[0051] Figure 9 shows the nucleotide sequence (SEQ ID NO: 16) and deduced amino acid sequence (SEQ ID NO: 2) of the *cry31Aa2* gene along with features thereof; and

[0052] Figure 10 shows the nucleotide sequence of the translated portion of the *cry31Aa1* gene (SEQ ID NO: 17).

DESCRIPTION OF THE PREFERRED EMBODIMENT

Isolation of strain, morphological and biochemical characteristics

[0053] A *Bacillus thuringiensis* strain was isolated from dead two-spotted spider mites (*Tetranychus urticae* Koch; Arthropoda: Arachnida: Tetranychidae) and named M15. The mites, parasitic on apple leaves, were collected in an apple orchard located in Frelighsburgh, Quebec, Canada. They were homogenized in 3 ml of phosphate-buffered saline (PBS) (NaCl 8 g, KCl 0.2 g, Na2HPO4 1.44 g, KH2PO4 0.24 g l-1). The homogenized solution was incubated for 16 hr at room temp and heated at 78°C for 15 min. Afterwards, the homogenate was plated on 2YT agar medium (Bacto Tryptone 16 g, Bacto Yeast Extract 10 g, NaCl 5 g, Agar 18 g l-1), and incubated for 24 hr at 30°C. All colonies with a morphology similar to *B. thuringiensis* were streaked on T3 agar medium (Bacto Tryptone 3 g, Bacto Tryptose 2 g, Bacto Yeast Extract 1.5 g, MnCl2 0.005 g, 0.05M Sodium phosphate, pH6.7, Agar 18 g l-1) and incubated at 30°C for 48 hr. The cultures were examined by phase-contrast microscopy (Carl Zeiss Canada Ltd., Toronto, Ontario, Canada) for the presence of spores and crystals. *B. thuringiensis* M15 was deposited on 29 January 2001 in the International Depository Authority of Health Canada in Winnipeg under the Budapest Treaty (Bureau of Microbiology, Health Canada, 1015 Arlington Street, Winnipeg, Manitoba, R3E 3R2) under accession no.

IDAC010201-5.

[0054] The M15 strain was characterized for its ability to ferment specific carbon sources, and for the production, utilization and reduction of specific compounds (see Table 1 below). The biochemical characteristics of *B. thuringiensis* strain M15, obtained using the API 50CH and API 20E kits as recommended by the manufacturer (bioMérieux, St-Laurent, Quebec, Canada), were different from those of three controls, *B. thuringiensis* var. *kurstaki* HD-1, -var. *israelensis* HD-500 and -var. *higo* BT205. *B. thuringiensis* var. *kurstaki* HD-1 and -var. *israelensis* HD-500 were obtained from "Laboratoire des bactéries entomopathogènes", Institut Pasteur (Paris, France). *B. thuringiensis* var. *higo* BT205 was in the Agriculture Canada collection (Jung *et al.*, 1998). The M15 strain is further characterized in Jung, 2001.

Table 1. The biochemical profile of *B. thuringiensis* M15 and selected control strains

Tests	*B. thuringiensis* var. *kurstaki* HD-1	*B. thuringiensis* var. *israelensis* HD-500	*B. thuringiensis* var. *higo* BT 205	*B. thuringiensis* M 15
Fermentation of Glycerol	+	+	+	±
D-Arabinose	-	-	-	-
L-Arabinose	-	-	-	-
Ribose	+	+	+	+
D-Xylose	-	-	-	-
L-Xylose	-	-	-	-
D-Galactose	-	-	-	-
D-Glucose	+	+	+	+
D-Fructose	+	+	+	+
D-Mannose	-	-	-	-
L-Sorbose	-	-	-	-
Inositol	-	-	-	-
D-Mannitol	-	-	-	-
D-Sorbitol	-	-	-	-
N-Acetylglucosamine	+	+	+	+
Arbutin	+	+	+	-

Esculin	+	±	+	±
Salicin	+	-	+	+
D-Cellobiose	+	+	+	-
D-Maltose	+	+	+	+
Lactose	-	-	-	-
Melibiose	-	-	-	-
Sucrose	-	-	-	-
Trehalose	+	+	+	+
Starch	-	+	+	-
Glycogen	+	+	+	-
Gluconate	+	+	±	-
Production of β-Galactosidase	-	-	-	-
Arginine dihydrolase	+	+	+	-
Ornithine decarboxylase	-	-	-	-
Urease	+	-	+	+
Tryptophane deaminase	-	-	-	-
Gelatinase	+	+	+	+
Oxidase	+	+	+	+
Catalase	+	+	+	+
H_2S	-	-	-	-
Indole	-	-	-	-
Acetoin	+	+	+	+
Citrate utilization	+	-	-	-
Nitrate reduction	+	-	-	-

+, -, and ± indicate positive, negative, and weak reactions, respectively.

Microscopic characterization of Cry31Aa2 parasporal inclusion bodies

[0055] The parasporal inclusion bodies produced by a sporulated culture of *B. thuringiensis* strain M15 appear roughly spherical when observed under phase-contrast microscopy (Fig. 1A) and are tightly coupled to the spores even in lysed cultures. Further analysis under the transmission electron microscope (TEM), however, reveals that the parasporal inclusion body has a polygonal

shape (Fig. 1B). The TEM observation was conducted after the *B. thuringiensis* strain M15 was incubated for 5 days at 30°C in T3 medium and the samples ultra-thinly sectioned according to Beveridge *et al.* (1994). Arrows show the roughly spherical parasporal inclusions tightly bound to the white ovoid spores. In this figure, "S" and "P" denote spore and parasporal inclusion, respectively. Magnification used is of 25,000 X.

SDS-PAGE analysis and N-terminal sequencing of the native parasporal inclusion protein

[0056] The *B. thuringiensis* strain M15 was grown in T3 medium for 5 days at 30°C on a rotary shaker to allow crystal protein production. Spores and crystals were separated from each other in the tightly bound parasporal duplexes using an ultrasonic processor model VC130 (Sonics & Materials, Inc., Newtown, CT, USA) and purified by sucrose density gradient centrifugation as described elsewhere (Thomas and Ellar, 1983). Twenty microliters of the crystal suspension were added to 3 volumes of gel loading buffer (4% SDS, 20% glycerol, 125 mM Tris-HCl, 10% 2-mercaptoethanol, pH 6.8) in a 1.5-ml microtube, incubated at 90°C for 7 min and centrifuged for 2 min to remove unsolubilized materials. Thirty microliters of the supernatant were loaded on top of 10% SDS-polyacrylamide gels. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli and Favre (1973).

[0057] Figure 2 shows the *B. thuringiensis* strain M15 parasporal inclusion purified by sucrose density gradient centrifugation as subjected to a 10% SDS-PAGE electrophoresis (lane 4); the crude extracts of the fully lysed *B. thuringiensis* var. *kurstaki* HD-1 subjected to electrophoresis on the same gel (lane 3) as a control; and high molecular (lane 1) and low molecular masses

(lane 2) of standard protein markers on the left. At least two major bands of approximately 86- and 79-KDa in size were revealed. They were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad™), excised and subjected to a pulsed liquid phase sequencer for determination of N-terminal amino acid sequence.

[0058] The N-terminal amino acid sequence of the crystal protein *from B. thuringiensis* strain M15 was determined as follows. The purified parasporal crystal was added into 0.1N NaOH-3M HEPES solution and solubilized in 10 volumes of gel loading buffer by incubating in boiling water for 5 min. The crystal protein was separated on 10 % SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Mississauga, Ontario, Canada). The crystal protein band stained with Coomassie brilliant blue™ R-250 (Bio-Rad) was excised and subjected to a pulsed liquid phase sequencer model 473A (Applied Biosystems, Foster City, CA, USA) at the Regional Sequencing Facility (Centre de recherche du Centre hospitalier de l'Université Laval, Quebec, Canada).

[0059] The N-terminal sequence analysis revealed that both polypeptides (86- and 79-KDa) shared identical 20-amino acids residues. These were Met, Asp, Pro, Phe, Ser, Asn, Tyr, Ser, Glu, Gln, Lys, Tyr, Pro, Asp, Ser, Asn, Asn, Asn, Gln and Glu (SEQ ID NO: 3).

Southern hybridization and gene cloning

[0060] An 18-mer oligonucleotide sequence, referred to as M15-M, was deduced from a middle portion of the N-terminal amino acid sequence (Glu, Gln, Lys, Tyr, Pro, Asp (SEQ ID NO: 4)) of the 86-kDa crystal protein. The M15-M oligonucleotide was labeled by the Digoxigenin (DIG) oligonucleotide 3'-

end labeling kit containing DIG-11-ddUTP (Roche, Laval, Quebec, Canada) as recommended by the manufacturer. The labeled oligonucleotide was precipitated with 0.1 volume of 4M LiCl and 2.5 volumes of ice-cold ethanol, and transferred at -70°C for 30 min. The reaction was centrifuged at 16,000g for 15 min at 4°C. The washed pellet was resuspended in nuclease-free water, and stored at -20°C until use.

[0061] The M15-M generated had the following sequence: 5'-GARCARAARTAYCCNGAY-3' (SEQ ID NO: 5).

[0062] *B. thuringiensis* strain M15 was grown in Luria-Bertani (LB) medium (Bacto Tryptone 10 g, Bacto Yeast Extract 5 g, NaCl 5 g l-1) at 30°C for 16 hr on a rotary shaker. Plasmid DNA was isolated using the alkaline extraction method as described elsewhere (Birnboim and Doly, 1979) with the following modifications. Lysozyme™ (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) was added at a concentration of 2 mg.ml-1 and the cell suspension was incubated at 37°C for 1hr.

[0063] The plasmid DNA was then purified with Wizard™ Plus SV minipreps DNA purification system following the manufacturer's recommendation (Promega, Nepean, Ontario, Canada). Three samples of the plasmid were then digested with *Hin*dIII, *Hin*dIII/*Eco*RI and *Eco*RI (Gibco BRL), respectively, electrophoresed on a 0.7% agarose gel and transferred onto a Nytran™ nylon membrane (Schleicher & Schuell, Keene, New Hampshire, USA) by the method of Southern (1975). They were then probed with the DIG-labeled 18-mer M15-M oligonucleotide.

[0064] This Southern blot hybridization was performed using the DIG-

labeled oligonucleotides with the standard hybridization solution (5X SSC, 1% blocking reagent (Roche), 0.1% N-lauroylsarcosine, 0.02% SDS) for 13 hr at 39°C. After hybridization, the membrane was washed twice for 15 min each in 4X wash solution (4X SSC, 0.1% SDS) at 39 °C. Following the washes, detection of signals on the membrane was performed with the color-substrate solution containing NBT (4-Nitroblue tetrazolium chloride, Roche) and BCIP (5-Bromo-4-chloro-3-indolyl-phosphate, Roche) as recommended by the manufacturer. After hybridization and post-hybridization washes at 39°C, the M15-M probe strongly hybridized to an 8-kb HindIII, a 2.6-kb *Hin*dIII/*Eco*RI, and a 2.6-kb *Eco*RI fragment.

[0065] The purified *B. thuringiensis* M15 plasmid DNA was digested with HindIII and ligated with the HindIII-digested SAP (Shrimp Alkaline Phosphatase, Roche)-treated pBluescript™ II KS(+) (Stratagene, La Jolla, California, USA). After ligation, the recombinant DNA was transformed into *E. coli* DH5α (Gibco BRL, Burlington, Ontario, Canada). Preparation of *E. coli* DH5α competent cells and transformation were done as described (Sambrook et al., 1989).

[0066] The transformants were grown on LB agar plates containing 100 μg ml-1 ampicillin (Sigma-Aldrich Canada Ltd.) and 40 μg ml-1 X–Gal (5'-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Sigma-Aldrich Canada Ltd.) at 37°C. White colonies were toothpicks-transferred to 1 ml of fresh LB media supplemented with 100 μg ml-1 ampicillin, and incubated overnight at 37°C.

[0067] The recombinant DNA were then isolated by the cracking procedure (Sambrook *et al.*, 1989) and electrophoresed on 0.7% agarose gel to assess the size of the undigested recombinant plasmids.

[0068] The three recombinant plasmids with the highest molecular weight were selected and digested with *Hin*dIII. They were designated pYCH27, pYCH40 and pYCH217, respectively. All three plasmids contained an 8-kb *Hin*dIII insert. In addition, pYCH27 and pYCH40 also contained a 0.75-kb and a 1.9-kb *Hin*dIII fragment, respectively. They were then electrophoresed on a 0.7% agarose gel, transferred onto a Nytran™ nylon membrane by the method of Southern (1975) and probed with the M15-M oligonucleotide. The M15-M probe hybridized to the 8-kb *Hin*dIII fragments in pYCH27, pYCH40 and pYCH217 as revealed by Southern blot hybridization.

[0069] The 8-kb *Hin*dIII fragments from pYCH27, pYCH40 and pYCH217 were doubly digested with *Hin*dIII/*Eco*RI, electrophoresed on agarose gel, Southern transferred, and hybridized with the M15-M probe. For each of the three recombinant plasmids, a single 2.6-kb fragment was detected (data not shown). This confirms that this 2.6-kb fragment is the same as the one in the *Eco*RI-digested plasmid DNA of strain M15.

[0070] The 8-kb *Hin*dIII insert was excised from recombinant plasmid pYCH217, digested with various restriction enzymes [*Eco*RI, *Bgl*II (Gibco BRL), *Dra*I, *Sph*I (Amersham Pharmacia Biotech)], and a restriction map constructed. The 8-kb *Hin*dIII fragment contains a 3.4-kb *Hin*dIII/*Eco*RI, a 2.6-kb *Eco*RI/*Eco*RI, a 1.4-kb *Eco*RI/*Eco*RI and a 0.6-kb *Eco*RI/*Hin*dIII fragment.

[0071] To identify the region homologous to the M15-M probe, the recombinant plasmid pYCH217 was doubly digested with HindIII/EcoRI, and the resulting fragments were subcloned into *Eco*RI-digested pBluescript™ II KS(+). After ligation, four subclones were obtained to give the recombinant plasmids pYC12S, pYC22S, pYC30S, and pYC31S. Plasmids pYC12S and pYC30S contained a 1.4-kb and a 2.6-kb insert, respectively, while pYC22S

and pYC31S both harbored a 2.6-kb insert along with a 0.6-kb and a 1.4-kb fragment, respectively. Only the 2.6-kb EcoRI/EcoRI fragment from subclones, pYC22S, pYC30S and pYC31S hybridized with the M15-M probe. To further localize the region of hybridization of the M15-M probe in the 2.6-kb EcoRI/EcoRI fragment, the recombinant plasmid pYC30S was digested with EcoRI, EcoRI/DraI, EcoRI/SphI, and EcoRI/BglII, respectively, and then hybridized with the M15-M probe. The M15-M probe detected a 2.6-kb EcoRI, a 0.6-kb DraI, a 1.6-kb EcoRI/SphI, and a 0.85-kb EcoRI/BglII fragment, respectively. It was thus determined that the region of hybridization of the M15-M probe lied between the BglII and DraI sites within the 2.6-kb EcoRI fragment.

Characterization of a new crystal protein gene, *cry31Aa2*

[0072] The nucleotide sequences of the 2.6-kb EcoRI/EcoRI, 1.4-kb EcoRI/EcoRI and 0.6-kb EcoRI/HindIII fragments were determined. An open reading frame (ORF) of 2,226-bp in length that codes for a polypeptide of 742 amino acids with a predicted molecular mass of 83,068Da (Figures 3 and 4) was found. The start codon is not ATG but GTG. One potential promoter-like sequence in the 5' non-coding region (Lereclus *et al.*, 1989; Baum and Malvar, 1995) shows a 13-bp spacing between the putative -10 and -35 sequences located 138-bp upstream from the start codon (GTG). The potential ribosome binding site (RBS) (GAAAGGTGG (SEQ ID NO: 6)) is located 7-bp upstream of the start codon (GTG) and is partially complementary to the 3' end (UCUUUCCUCC (SEQ ID NO: 7)) of *B. subtilis* 16S rRNA (McLaughlin *et al.*, 1981; Moran *et al.*, 1982). Both potential -35 and -10 boxes and a putative ribosome-binding site are underlined in figure 9. The calculated free energy of interaction (ΔG, 25°C) between the *B. subtilis* 16S rRNA and the putative ribosome binding site is -14.8 kcal·mol-1 (Tinoco et al., 1973). A terminal inverted repeat that could form a stem-and-loop secondary structure with a

calculated energy (ΔG, 25°C) of -12.2 kcal·mol-1 (Tinoco et al., 1973) is located 112-bp downstream from the stop codon (TAA), which is marked with asterisks in figure 9, and may function as a transcription terminator (indicated by arrows). The 18-mer M15-M oligonucleotide sequence based on the N-terminal amino acid sequence (Glu, Gln, Lys, Tyr, Pro, Asp (SEQ ID NO: 4)) of the crystal protein is homologous to a region located 24-bp downstream from the start codon (GTG). The sequence of the DIG-labeled 18-mer oligonucleotide (M15-M) probe is indicated in bold capital letters in figure 9.

The *cry31Aa2* gene expression in *B. thuringiensis* Cry– B strain

[0073] The 3.6-kb HindIII/SphI fragment containing the entire crystal protein gene was excised from the recombinant plasmid pYCH217, and then cloned into the E. coli-*B. thuringiensis* shuttle vector pHPS9 doubly digested with HindIII/SphI to yield recombinant plasmid pYCP31A2 (Figure 6). The *E. coli-B. thuringiensis* shuttle vector pHPS9 (Haima, et al., 1990) was purchased from American Type Culture Collection (Manassas, VA, USA). To express the cloned *cry31Aa2* crystal protein gene in the acrystalliferous *B. thuringiensis* strain Cry– B, the 3.6-kb HindIII/SphI fragment was cloned into the HindIII/SphI doubly-digested E. coli-*B. thuringiensis* shuttle vector pHPS9 to yield recombinant plasmid pYCP31A2 (Fig. 6).

[0074] The *B. thuringiensis* var. *kurstaki* HD-1 acrystalliferous Cry–B strain ((Stahly et al., 1978) provided by the *Bacillus* Genetic Stock Center, The Ohio State University (Columbus, OH, USA)), was transformed with the cloned *B. thuringiensis* M15 crystal protein gene by electroporation as described by Vehmaanperä (1989) with the following modifications. Bacterial cells cultured in 200 ml of LB supplemented with 0.25 M sucrose and 0.05 M potassium phosphate, pH7.0 (LBSP) to an optical density of 1.0 at 600 nm were

centrifuged, washed three times with ice-cold SHMG buffer (250 mM sucrose, 1 mM HEPES, 1 mM MgCl2, 10% (v/v) glycerol, pH 7.0), and then resuspended in 1 ml of ice-cold SHMG buffer. The cell suspension was mixed with plasmid DNA at a final DNA concentration of 10 μg ml-1 in a 0.2-cm electroporation cuvette (Bio-Rad), kept on ice for 30 min, and then electroporated by a Gene PulserTM model 1652076 (Bio-Rad) at 25μF, 2.5kV and 400Ω with the pulse once. After electroporation, 3ml of LBSP supplemented with 10% (v/v) glycerol (LBSPG) were immediately added into the cuvette and incubated at 37°C for 2 hr with shaking.

[0075] The selected *B. thuringiensis* transformant was cultured in 250 ml of nutrient broth supplemented with 5 μg ml-1 of erythromycin (Sigma-Aldrich Canada Ltd.) and 5 μg ml-1 of chloramphenicol (Sigma-Aldrich Canada Ltd.) at 37°C until cell autolysis was observed. The lysate was harvested and then washed twice with 10 mM EDTA (pH 8.0)-1 M NaCl-1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich Canada Ltd.).

[0076] The *B. thuringiensis* Cry– B transformant containing the *B. thuringiensis* M15 parasporal crystal protein gene was incubated in nutrient broth (Bacto Beef Extract 3 g, Bacto Peptone 5 g l-1) at 30°C for 3 days to allow expression of the toxin gene and crystal formation. The presence of parasporal inclusions was examined by phase-contrast microscopy. When observed under a phase-contrast microscope, the *B. thuringiensis* transformants expressing the cry31Aa2 gene contained, in addition to the spore, a roughly spherical inclusion, whereas no inclusions were found in the *B. thuringiensis* transformant harboring the non-recombinant shuttle vector pHPS9 alone (data not shown). Under the transmission electron microscope (TEM), however, the parasporal inclusion body has a nearly perfect hexagonal shape (Fig. 7). Both inclusions in the transformant, spore and crystal, are separated

from each other as opposed to what is found in *B. thuringiensis* strain M15 where they are tightly bound to each other.

[0077] The parasporal inclusion from a *B. thuringiensis* transformant was purified by sucrose density gradient centrifugation as described previously (Thomas and Ellar, 1983). It was then subjected to a 10% discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (lane 3) as reported previously (Laemmli and Favre, 1973). High molecular (lane 1) and low molecular masses (lane 2) of standard protein markers are indicated on the left. The parasporal inclusion protein in the *B. thuringiensis* transformant is composed of a single major polypeptide of 83-kDa (Fig. 8).

Preparation of inclusion proteins, proteolytic processing, and toxin activation

[0078] The spore-inclusion mixture was harvested from sporulated cultures and the inclusions were partially purified by a biphasic separation method described in Goodman (1967) using polyethylene glycol 6000 (Wako Pure Chemical, Osaka, Japan) and sodium dextran sulfate 500 (Sigma, St. Louis, Mo.). Inclusions were further purified by sucrose density gradient centrifugation as described in Saitoh et al., (1998a). The purified inclusions were stored at 20°C until use.

[0079] Solubilization of purified inclusions was done in 50 mM Na_2CO_3 (pH 10.0) containing 1 mM EDTA and 10 mM dithiothreitol for 1 h at 37°C. After centrifugation at 20,000 × g for 5 min at 4°C to remove unsolubilized materials, the pH of the solution was adjusted to 8.0.

[0080] The native 83KDa protoxine displayed no cytocidal activity

against cancer cells. This protein was therefore cleaved with three enzymes, namely trypsin, chymotrypsin and proteinase K to identify an active toxine. The solubilized proteins (1.3 mg ml1) were therefore treated with proteinase K (final concentrations, 0.0003, 0.003, 0.03, and 0.3 mg ml1), trypsin (0.03, 0.3, 3, and 30 mg ml1), and chymotrypsin (0.03, 0.3, 3, and 30 mg ml1) in 50 mM Na2CO3 (pH 10.0) for 1.5 h at 37°C. After protease treatment, phenylmethylsulfonyl fluoride (Wako Pure Chemical) was added to the solution to stop the proteolytic reaction, and the mixture was examined for both sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles and cytopathic effect (CPE) on certain cancer cells including MOLT-4 and Hela. The CPE was monitored under a phase-contrast microscope for 24 h, and the degree of cytopathy was graded on the basis of the ratio of damaged cells as described in Mizuki *et al.*, (1999).

One-dose assay, hemolytic assay, and dose-response study

[0081] One-dose assays for cytotoxicity and hemolytic activity were carried out as described in Mizuki *et al.*, (1999). Each well of a MicroTest plate received 90 µl of cell suspension containing 2×10^4 cells. After preincubation for 16 h at 37°C, 10 µl of the trypsin-activated sample solution (1.3 mg ml) was added to the well.

[0082] Thirteen human cells, two monkey cells and one mouse cell were used for dose-response studies. A hemolytic assay was done using human erythrocytes according to the method described in Saitoh et al., (1998b). Each well containing 90 µl of cell suspension (2×10^4 cells) received 10 µl of trypsin-activated inclusion proteins which had been prepared in 10-fold serial dilutions in 50 mM Na_2CO_3 (pH 10.0) containing 10 mM DTT and 1 mM EDTA. Five wells were used for each dilution, and the test was repeated at least three

times. The CPE was monitored under a phase-contrast microscope at appropriate intervals for 24 h postinoculation.

[0083] For assessment of the level of cytotoxicity, a cell proliferation test using an MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide] assay as described in Behl (1992) and Heiss (1997) was conducted 24 h postinoculation by using a Premix™ WST-1 kit (Takara Co.). The average of absorbance in mock-inoculated negative controls was used as a blank value. The arbitrary unit was defined on the basis of the relative value of absorbance at 450 nm to the blank (1.0). The 50% effective concentrations (EC50s) were deduced from the dose-response curves using a log-probit program. Table 2 below presents the EC50s.

[0084] The protein exhibited cytotoxicity against HeLa, TCS, HL-60, Jurkat and Hep-G2 cells when treated with trypsin. No cytocidal activity was induced after treatment with chymotrypsin or proteinase K on HeLa, MOLT-4 and Sawano cells. Without protease digestion, inclusion proteins showed no cytocidal toxicity. Trypsin cleaves the cry31Aa2 protein after the arginine at position 250 of the full native protein sequence (SEQ ID NO: 2). The sequence of the trypsin-activated protein is designated SEQ ID NO: 8 and the corresponding nucleotide sequence is designated SEQ ID NO: 9.

[0085] Table 2 shows the results of one-dose assays of trypsin-activated Cry31Aa2 as compared to those of trypsin-activated Cry31Aa1 against several species of cultured cells. The toxicity spectrum of the protein from the recombinant Cry-B was similar to that of the protein of the wild strain M15. Both cloned proteins were highly or moderately cytocidal against HeLa, TCS, HL-60, Jurkat and Hep-G2 but were slightly toxic or nontoxic for normal T cells and for Sawano, UtSMC, MOLT-4, A549, MRC-5, HC, Caco-2 and the non

human cells tested.

Table 2 Effective concentration 50 of trypsin-activated *cry31Aa2* as compared to that of activated *cry31Aa1* on various cells

					EC50(ug/ml)	EC50(ug/ml)
		Organ	Cell	Cell type	Cry31Aa2	Cry31Aa1
1	Human	Uterus	HeLa	Cervix cancer	0.30	0.23
2			Sawano	Uterus cancer, adenocarcinoma	>10	
3			TCS	Cervix cancer, keratinizing squamous	0.32	
4			UtSMC	Uterus normal smooth muscle	>10	
5		Blood	MOLT-4	T cell leukaemia	>10	1.06
6			HL-60	T cell leukaemia	0.05	
7			Jurkat	T cell leukaemia	0.02	
8			T cell	Normal T cell	>10	
9		Lung	A549	Lung cancer	>10	
10			MRC-5	Lung normal fibroblast	>10	
11		Liver	HC	Normal hepatocyte cell	>10	
12			Hep-G2	Liver carcinoma hepatocellular	0.02	
13		Colon	Caco-2	Colon cancer, adenocarcinoma	>10	
14	Monkey	Kidney	Vero	Monkey, kidney epithelial cell	>10	
15			COS-7	Monkey, kidney SV40 transformed cell	>10	
16	Mouse	Embryo	NIH3T3	Mouse, embryo fibroblast cell	>10	

Comparison of Cry31Aa2 and Cry31Aa1

[0086] As may be seen in Figure 5, the Cry31Aa2 amino acid sequence shares extensive homology with Cry31Aa1 except for a substitution of 25 amino acid residues and an addition of 19 contiguous codons in *cry31Aa2* (Figure 5). This 19 amino acid sequence is as follows SYQNMKTEIVNTDLPYNTN and is designated SEQ ID NO: 10 while the corresponding nucleotide sequence is designated SEQ ID NO: 11. The asterisks under the Cry31Aa2 sequence indicate the identities between Cry31Aa2 and Cry31Aa1. The capital letters and dotted lines under the amino

acid sequence of Cry31Aa2 in Figure 5, correspond to the difference and alignment gaps between the Cry31Aa2 and Cry31Aa1 proteins. The 83-KDa Cry31Aa2 protein exhibits 94% amino acid sequence identities with the Cry31Aa1 protein. The five conserved amino acid blocks of the Cry31Aa2 protein were especially identical to those of the Cry31Aa1 protein except for the substitution of a single lysine residue in the second conserved block of Cry31Aa2. The bold lines above the Cry31Aa2 sequence correspond to the five conserved amino acid blocks found in the amino acid sequence of cry31Aa1. Both Cry31Aa2 and Cry31Aa1 show very low amino acid sequence homology to the known *B. thuringiensis* Cry and Cyt proteins (Mizuki *et al.*, 2000). The nucleotide sequence of the portion of the *cry31Aa2* gene encoding the trypsin-activated protein shares a 98% identity with the corresponding sequence of *cry31Aa1*.

[0087] Table 2 above shows that both Cry31Aa2 and Cry31Aa1 protein display cytotoxicity against a number of human cancer cells. The cytocidal activity of the Cry31Aa1 was due to the cleavage by proteinase K and trypsin (Mizuki *et al.*, 2000) while that of Cry31Aa2 was due to trypsin. The comparison of the amino acid sequence of Cry31Aa1 with that of Cry31Aa2 indicate which amino acids of the amino acid sequence of members of the Cry31 family can be substituted without abrogating this cytoxicity against human cancer cells. Although certain of these substitutions surely provide for the specificity of each of Cry31Aa2 and Cry31Aa1 against specific human cancer cells, they each display a significant toxicity against a number of human cancer cells. Hence, it is submitted that the amino acids at positions 24, 37, 39, 51, 56, 59, 87, 97, 138, 158, 170, 251, 389, 444-446, 466, 481, 507, 510, 518, 551, 582, 637, 725 and 742 can be replaced by any other amino acid without abrogating the cytotoxicity of the protein that it constitutes against at least some cancer cells. Sequences encompassing substitutions at these positions in the complete

Cry31Aa2 protein sequence (SEQ ID NO: 2) and in the trypsin-activated Cry31Aa2 protein sequence (SEQ ID NO: 8) starting after the arginine at position 250 are within the scope of the present invention and are designated herein as SEQ ID NOs: 12 and 13, respectively.

[0088] Certain substitutions are preferred however and correspond to either a substitution by an amino acid having similar chemical properties or, even more preferred, a substitution by the amino acid found at the corresponding position in Cry31Aa1. Amino acids are categorized herein into 5 groups of amino acids according to their chemical properties, namely small nonpolar (i.e. C, P, A and T), small polar (i.e. S, G, D and N), large polar (i.e. E, Q, K and R), intermediate polarity (i.e. Y, H and W), aand large nonpolar (i.e. F, M, L, I and V) . Hence, the amino acid at position 24 of the Cry31Aa2, is preferably a polar amino acid, most preferably a large polar amino acid and even more preferably glutamate or lysine. The amino acid at position 37 is preferably methionine or alanine. The amino acid at position 39 is preferably threonine or asparagine. The amino acid at position 51, is preferably a nonpolar amino acid, most preferably a small nonpolar amino acid and even more preferably alanine or threonine. The amino acid at position 56 is preferably proline or serine. The amino acid at position 59 is preferably an amino acid of intermediate polarity and most preferably tyrosine or tryptophan. The amino acid at position 87, is preferably a polar amino acid, most preferably a small polar amino acid and even more preferably asparagine or aspartate. The amino acid at position 97, is preferably a polar amino acid, most preferably a large polar amino acid and even most preferably arginine or lysine. The amino acid at position 138 is preferably a polar amino acid, most preferably a large polar amino acid and even more preferably glutamate or lysine. The amino acid at position 158 is preferably alanine or asparagine. The amino acid at position 170 is preferably a polar amino acid, most preferably a small polar amino acid and

even more preferably glycine or serine.

[0089] The amino acid at position 251 is preferably a nonpolar amino acid, most preferably a large nonpolar amino acid and even more preferably isoleucine or methionine. The amino acid at position 389 is preferably a polar amino acid, most preferably a large polar amino acid and even more preferably lysine or arginine. The amino acid at position 444 is preferably serine or histidine. The amino acid at position 445 is preferably a polar amino acid, most preferably a small polar amino acid and even more preferably glycine or serine. The amino acid at position 446 is preferably glycine or proline. The amino acid at position 466 is preferably a polar amino acid, most preferably a large polar amino acid and even more preferably glutamine or arginine. The amino acid at position 481 is preferably an amino acid of intermediate polarity and most preferably tyrosine or tryptophan. The amino acid at position 507 is preferably alanine or leucine. The amino acid at position 510 is preferably glycine or histidine. The amino acid at position 518 is preferably a nonpolar amino acid and is preferably alanine or valine. The amino acid at position 551 is preferably a nonpolar amino acid, most preferably a small nonpolar amino acid and even more preferably alanine or proline. The amino acid at position 582 is preferably a nonpolar amino acid, most preferably a small nonpolar amino acid and even more preferably alanine or threonine. The amino acid at position 637 is preferably arginine or isoleucine. The amino acid at position 725 is preferably glycine or arginine. Finally, the amino acid at position 742 is preferably valine or serine. Sequences encompassing the most preferred substitutions listed above at these positions in the complete Cry31Aa2 protein sequence (SEQ ID NO: 2) and in the trypsin-activated Cry31Aa2 protein sequence (SEQ ID NO: 8) starting after the arginine at position 250 are within the scope of the present invention and are designated herein as SEQ ID NOs: 14 and 15, respectively

[0090] Sequences encompassing all the possible substitutions to the *cry31Aa2* gene nucleotide sequence, the crystal protein and the trypsin-activated crystal protein derived from the crystal protein of the *Bacillus thuringiensis* M15 deposited under no. IDAC010201-5 as described above are within the scope of the present invention.

[0091] Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

REFERENCES

1. Aronson, A. (1993). The two faces of *Bacillus thuringiensis*: insecticidal proteins and post-exponential survival. *Mol. Microbiol.* **7**, 489-496.

2. Baum, J. A., and Malvar, T. (1995). Regulation of insecticidal crystal protein production in *Bacillus thuringiensis*. *Mol. Microbiol.* **18**, 1-12.

3. Behl, C., J. Davis, G. M. Cole, and D. Schubert. 1992. Vitamin E protects nerve cells from *amyloid* protein toxicity. *Biochem. Biophys. Res. Commun.* **186:**944-950.

4. Beveridge, T. J., Popkin, T. J., and Cole, R. M. (1994). Electron microscopy. *In* "Methods for General and Molecular Bacteriology" (Gerhardt, P., R. G. E. Murray, W. A. Wood, and N. R. Krieg, Eds.), pp. 42-71. Washington, D.C. Am. Soc. for Microbiol.

5. Birnboim, H. C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**, 1513-1523.

6. Crickmore, N. 2001. *Bacillus thuringiensis* toxin gene nomenclature. Web site http://epunix. biols. susx. ac. uk/Home/Neil_Crickmore/Bt/index.html.

7. Crickmore, N., Zeigler, D. R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., and Dean, D. H. (1998). Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **62**, 807-813. de Barjac, H., and Frachon, E. (1990). Classification of *Bacillus thuringiensis* strains. *Entomophaga* **35**, 233-240.

8. Feitelson, J. S., Payne, J., and Kim, L. (1992). *Bacillus thuringiensis*: insects and beyond. *Bio/Technology* **10**, 271-275.

9. Gill, S. S., Cowles, E. A., and Pietrantonio, P. V. (1992). The mode of action of *B. thuringiensis* endotoxins. *Annu. Rev. Entomol.* **37**, 615-636.

10. Goodman, N. S., R. J. Gottfried, and M. H. Rogoff. 1967. Biphasic system for separation of spores and crystals of *Bacillus thuringiensis*. *J. Bacteriol.* **94:**485

11. Haima, P., Van Sinderen, D., Schotting, H., Bron, S., and Venema, G. (1990). Development of a β-galactosidase α-complementation system for molecular cloning in *Bacillus subtilis*. *Gene* **86**, 63-69.

12. Heiss, P., S. Bernatz, G. Bruchelt, and R. Senekowitsch-Schmidtke. 1997. Cytotoxic effect of immunoconjugate composed of glucose-oxidase coupled to an anti-ganglioside (G_{D2}) antibody on spheroids. *Anticancer Res.* **17**:3177-3178

13. Höfte, H., and Whiteley, H. R. (1989). Insecticidal crystal proteins of *B. thuringiensis. Microbiol. Rev.* **53**, 242-255.

14. Jung, Y. C., Kim, S. U., Côté, J. -C., Lecadet, M. -M., and Chung, Y. S., and Bok, S. H. (1998). Characterization of a new *Bacillus thuringiensis* subsp. *higo* strain isolated from rice bran in Korea. *J. Invertebr. Pathol.* **71**, 95-96.

15. Kim, H. S. (2000). Comparative study of the frequency, flagellar serotype, crystal shape, toxicity, and *cry* gene contents of *Bacillus thuringiensis* from three environments. *Curr. Microbiol.* **41**, 250-256.

16. Laemmli, U. K., and Favre, M. (1973). Maturation of the head of acteriophage T4. *J. Mol. Biol.* **80**, 575-599.

17. Lecadet, M. -M., Frachon, E., Cosmao Dumanoir, V. *et al.* (1999). Updating the H-antigen classification of *Bacillus thuringiensis*. *J. Appl. Microbiol.* **86**, 660-672.

18. Lee, D. W., Akao, T., Yamashita, S., Katayama, H., Maeda, M., Saitoh, H., Mizuki, E., and Ohba, M. (2000). Noninsecticidal parasporal proteins of a *Bacillus thuringiensis* serovar *shandongiensis* isolate exhibit a preferential cytotoxicity against human leukemic T cells. *Biochem. Biophys. Res. Commun.* **272**, 218-223.

19. Lereclus, D., Bourgouin, C., Lecadet, M. -M., Klier, A., and Rapoport, G. (1989). Role, structure, and molecular organization of the genes coding for the parasporal δ-endotoxins of *B. thuringiensis. In* "Regulation of Procaryotic Development: Structural and Functional Analysis of Bacterial Sporulation and Germination" (Smith, I., R. A. Slepecky, and P. Setlow, Eds.), pp. 255-276, Washington, D.C. Am. Soc. for Microbiol.

20. McLaughlin, J. R., Murray, C. L., and Rabinowitz, J. C. (1981). Unique features in the ribosome binding site sequence of the Gram-positive Staphylococcus aureus β-lactamase gene. *J. Biol. Chem.* **256**, 11283-11291.

21. Mizuki, E., Ohba, M., Akao, T., Yamashita, S., Saitoh, H., and Park, Y. S. (1999). Unique activity associated with non-insecticidal *Bacillus thuringiensis* parasporal inclusions: *in vitro* cell-killing action on human

cancer cells. *J. Appl. Microbiol.* **86**, 477-486.

22. Mizuki, E., Park, Y. S., Saitoh, H., Yamashita, S., Akao, T., Higuchi, K., and Ohba, M. (2000). Parasporin, a human leukemic cell-recognizing parasporal protein of *Bacillus thuringiensis. Clin. Diag. Lab. Immunol.* **7**, 625-634.

23. Moran, C. P., Lang, N., Jr., LeGrice, S. F. J., Lee, G., Stephens, M., Sonenshein, A. L., Pero, J., and Losick, R. (1982). Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis. Mol Gen. Gene*. **186**, 339-346.

24. Ohba, M. (1996). *Bacillus thuringiensis* populations naturally occuring on mulberry leaves: a possible source of the populations associated with silkworm-rearing insectaries. *J. Appl. Bacteriol.* **80**, 56-64.

25. Ohba, M., and Aizawa, K. (1986). Insect toxicity of *Bacillus thuringiensis* isolated from soils of Japan. *J. Invertebr. Pathol.* **47**, 12-20.

26. Roh, J. Y., Park, H. W., Jin, B. R., Kim, H. S., Yu, Y. M., and Kang, S. K. (1996). Characterization of novel non-toxic *Bacillus thuringiensis* isolates from Korea. *Lett. Appl. Microbiol.* **23**, 249-252.

27. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular cloning. A Laboratory manual," 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

28. Saitoh, H., K. Higuchi, E. Mizuki, and M. Ohba. (1998a). Larvicidal toxicity of Japanese *Bacillus thuringiensis* against the mosquito *Anopheles stephensi. Med. Vet. Entomol.* **12**:98-102.

29. Saitoh, H., K. Higuchi, E. Mizuki, S.-H. Hwang, and M. Ohba. (1998b). Characterization of mosquito larvicidal parasporal inclusions of a *Bacillus thuringiensis* serovar *higo* strain. *J. Appl. Microbiol.* **84**:883-888

30. Schnepf, H. E., Wong, H. C., and Whiteley, H. R. (1985). The amino acid sequence of a crystal protein from *Bacillus thuringiensis* deduced from the DNA base sequence. *J. Biol. Chem.* **260**, 6264-6272.

31. Southern, E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**, 503-517.

32. Stahly, D. P., Dingman, D. W., Bulla, L. A., Jr., and Aronson, A. I. (1978). Possible origin and function of the parasporal crystal in *Bacillus thuringiensis. Biochem. Biophys. Res. Commun.* **84**, 581-588.

33. Thomas, W. E., and Ellar, D. J. (1983). *B. thuringiensis* var. *israelensis* crystal δ-endotoxin: effects on insect and mammalian cells *in vitro* and *in vivo*. *J. Cell. Sci.* **60**, 181-197.

34. Tinoco, I., Jr., Borer, P. N., Dengler, B., Levine, M. D., Uhlenbeck, O. C., Crothers, D. M., and Gralla, J. (1973). Improved estimation of secondary structure in ribonucleic acids. *Nature New Biol.* **246**, 40-41.

35. Vehmaanperä, J. (1989). Transformation of *Bacillus amyloliquefaciens* by electroporation. *FEMS Microbiol. Lett.* **61**, 165-170.

36. Jung, Y.-C. (2001) *Bacillus thuringiensis* Strain M15, a Novel Autoagglutinable, Non-Serotypeable Strain-Cloning and Characterization of a Novel *cry31A*-type Crystal Protein Gene, *cry31Aa2*, and Two New Insertion Sequences, IS*231*M and –N. Département de sciences biologiques, Faculté des arts et des sciences, Université de Montréal.